Effect of Adolescent Chronic Mild Stress and Npas4 Deficiency on the Prefrontal Cortex and Prefrontal-Dependent Cognitive Functions

Author: Jonathan Alexander

The Ohio State University Department of Neuroscience

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Mentor: Dr. Laurence Coutellier

Department of Neuroscience and Psychology

coutellier.8@osu.edu

Abstract

Chronic stress exposure in adolescence has been shown to impair cognitive function, especially in individuals with genetic vulnerabilities. Despite this, there is little known about what mechanisms are underlying how these genetic predispositions and environmental factors present in adolescence cause cognitive impairments. During adolescence, the GABAergic system of the prefrontal cortex (PFC) undergoes a spike in development that may leave the area vulnerable to these kind of genetic and environmental factors, thus contributing to observed cognitive changes. Npas4 is well established as an important protein relating to plasticity and inhibitory synaptogenesis in the hippocampus and cortical areas, and Npas4 expression has been found to be suppressed by general stress and corticosterone, correlating to significant memory, cognitive, and social deficits in mouse models. Furthermore, our lab has recently demonstrated that Npas4 plays a significant role in adolescent prefrontal maturation. Together, these findings suggest that Npas4 might be a mediator of adolescent stress exposure on prefrontal-dependent impairments. In this study, we aimed to uncover the mechanisms by which Npas4 modulates the effects of stress on cognitive functions and brain structures. We hypothesized that Npas4 under-expression and exposure to chronic mild stress (CMS) in adolescence would produce PFC-derived behavioral changes and abnormalities in brain structures after development. To this end, we developed a mouse model demonstrating the effects produced by Npas4 knockdown and adolescent stress on relevant cell populations and myelination in the prefrontal cortex and prefrontal-dependent cognitive function.

We compared Npas4 wildtype and heterozygous mice, chronically stressed in adolescence, and assessed PFC-dependent cognitive functions, including cognitive flexibility

through the attention set shift task. mRNA levels of important biomarkers of myelin and the cell death process in the PFC were measured using RT-qPCR, and the number of neurons and oligodendrocytes, and myelination density in the ventromedial PFC (vmPFC) as quantified through immunohistochemistry (IHC) to evaluate cell death and myelination loss in the prefrontal cortex. We observed a trend towards cognitive flexibility impairment through the extradimensional shift phase of the attention set shift task and a trend towards reduced mRNA expression of the myelin basic protein (MPB) along with significant changes in PLP/DM20 and Caspase-3 in heterozygous Npas4 mice exposed to chronic stress. This data did not correlate with a reduction in neuron or oligodendrocyte populations, and there were no marked changes in myelination density. Discovering if the interaction between stress-exposure and Npas4 deficiency in adolescent mice produce any structural changes in the PFC would help progress the understanding of how stress in adolescence affects the developing brain. From here, other factors could be determined and structural changes identified. Through these, methods of identification of susceptible individuals and potential interventions could be assessed.

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Introduction

Background:

Chronic stress exposure has been identified as a risk factor for a myriad of psychiatric disorders, with vulnerability to stress spiking in the formative span of adolescence (Buwalda *et al.,* 2011). Many psychological disorders have been found in association with improper development of frontal cortex structures and white matter connections during this period. Specifically, schizophrenia has been associated with changes in white matter paths and overall density and the balance of inhibition and excitation is skewed in depression (Pittenger & Duman, 2007) (Lewis, 1997) (Kubicki *et al.,* 2009). Despite this, not every stressful adolescence produces a psychiatrically diagnosable adult. Genetics have been solidly established as determiners of psychological disease and stress response as well, but specifically how genetics and stress interact mechanistically to produce structures and behavior analogous with these disorders is not well understood.

The Npas4 gene is a member of the helix-loop-helix PAS domain-producing group of genes that are responsible for neuronal responses to environmental changes. These proteins are specifically responsible for functioning as sensors of change and modes of response and plasticity in the nervous system; stimuli activating this domain include such things as oxygen concentration, redox potential, light, and the presence of certain ligands (Gu *et al.,* 2000). PAS domains typically exist in the cytoplasm –which is uncommon for sensor proteins –but may also respond to activity outside of the cell membrane, allowing them to react to changes both in the intracellular environment as well as the environment outside of the cell (Taylor & Zuhlin, 1999).

Specifically, Npas4 functions as a transcription factor that responds to an influx of calcium resulting from an excitatory signal to upregulate inhibitory synapses on the cell body and dendrites of excitatory neurons (Lin *et al.,* 2008). This is an example of activity-regulated long term potentiation (LTP) at these inhibitory synapses. Notably, the balance between excitatory and inhibitory inputs is necessary to represent sensory information and maintain plasticity (Kenet *et al.,* 2007), control motor outputs (Brown *et al.,* 1996), and execution of complex cognitive functions. Npas4 has been shown to be proliferative not only in high amounts in the limbic system, but also in the parietal and, notably for this study, the frontal cortex (Damborsky *et al.,* 2015; Coutellier *et al.,* 2015). In fact, during adolescence, Npas4 is significantly higher in concentration in the PFC than any other point (Damborsky *et al.,* 2015).

Npas4 expression and function has been linked to stress exposure and corticosterone levels in the dentate gyrus of the hippocampus; mRNA of the gene is significantly reduced in instances of acute and chronic stress (Yun *et al.,* 2010; Coutellier *et al.,* 2015). It has also been shown to negatively affect prefrontal-associated behaviors in mice models exposed to chronic stress (Coutellier *et al.,* 2015). Reduced expression of this gene has the potential to disrupt the balance of inhibition and excitation in the key areas that it mediates, especially when influenced by stressors that down-regulate its expression.

Purpose:

Previous research in this lab found significant cognitive deficits in behavioral tests classically involving the prefrontal cortex in Npas4 deficient mice exposed to chronic stress in adolescence. In order to better understand how Npas4 may mediate the stress response in areas of

the brain associated with cognitive functions, we attempted to quantify structural differences present in the prelimbic and infralimbic cortexes of the ventromedial prefrontal cortex (vmPFC) that may explain the behavioral changes that are well established as a result of adolescent stress and a deficiency in expression. Initial RT-qPCR data presented compelling trends in markers of general cell apoptosis and general myelin deficiency. This directed the rest of the study to attempt to correlate these results with a decrease in neuron or oligodendrocyte populations, or less dense myelin coverage in this area. Results here would provide a compelling mechanism by which stress and genetics could change the outcome of prefrontal cortex structures and perhaps allow a way to prevent these changes in at-risk individuals for associated psychological disorders.

The study specifically sought to identify how Npas4 modulated stress vulnerability in adolescence when PFC structures are still developing. We hypothesized that Npas4 deficiency and adolescent stress would interact to affect neuronal or glial populations in the developing vmPFC, and would lead to a corresponding decline in PFC-derived cognitive deficits.

Materials and Methods

Animals:

Npas4 transgenic mice were used to determine the effect of Npas4 on stress vulnerability. For all portions of the study, male Npas4 wild-type (WT) and heterozygous (HET) mice – expressing about 50% of the normal Npas4 level –were obtained via HETxHET breeding. Mice were group-housed until chronic mild stress (CMS) procedure and kept on a 12-hour reverse light and dark cycle. Food and water was provided with no restriction unless otherwise noted.

We utilized a 2x2 model using the Npas4 genotype (HET and WT mice), and the adolescent environment (stressed and unstressed) as our independent variables. For each, n=3-9 mice per genotype/treatment group. Four cohorts were used in the RT-qPCR, neuron and oligodendrocyte immunohistochemistry, myelin quantification, and behavioral analysis.

In the first two cohorts, the non-stress group underwent standard rearing throughout adolescence (PND28 to PND42) and, after which, were euthanized. In the stress group, mice underwent CMS and were euthanized during the same timeframe, 90 minutes following the final CMS procedure. One cohort was used for RT-qPCR and the second for neuron and oligodendrocyte immunohistochemistry.

The third and fourth cohorts followed the same outline as above for CMS or standard rearing timeline, but mice were kept until adulthood when they were tested for their PFC-dependent cognitive function as well as to quantify myelination density respectively.

Chronic Mild Stress Procedure:

Experimental Npas4 mice were bred and the date of birth recorded as PND0. After weaning at PND21, mice were group-housed based on sex. Mice undergoing CMS procedure were socially isolated (SI) along with forced swim stress (FSS) every other day throughout the two weeks defined as adolescence (PND28 to PND42). On day one, CMS mice were changed to single housing without shelter items. For the FSS, mice were placed in a glass cylinder (of height 30cm; diameter 15cm) filled with water (24-25˚C) for a period of 4 minutes. Following this, mice recovered on a heated pad in their home cage until dry. For the neuron and oligodendrocyte quantification cohort, perfusion commenced 90 minutes following the final swim or similar timeframe in the unstressed groups. The RT-qPCR group was flash frozen at baseline. The other two cohorts were left undisturbed after adolescence except for standard care procedure until adulthood where behavioral testing and utilization for myelination analysis occurred.

Mice that were used in RT-qPCR were anesthetized with isoflurane, quickly decapitated, and brains were flash-frozen with dry ice. These brains were stored at -80˚C. All other cohorts were anesthetized with isoflurane and perfused transcardially using 0.1 M phosphate-buffered saline at pH 7.4 then 4% PFA. These brains were removed and placed overnight in PFA at 4˚C, after which they were allowed to saturate in 30% sucrose until sinking.

Quantification of Biomarkers and Markers of Cell Apoptosis in the PFC:

In order to determine differences in indicators of PFC structure, Npas4 HET and WT mice underwent standard rearing or CMS procedure as outlined above and were thereafter

euthanized and flash frozen at baseline. Dissection proceeded in a cold room on dry ice. PFC containing regions were collected based on the Franklin and Paxinos mouse brain atlas.

RT-qPCR was performed on PFC structures using primers for Caspase-3, MBP, and PLP/DM20. Caspase-3 is a tissue specific protease involved with cell apoptosis (Porter & Jänicke, 1999). MBP is important for the formation of the major dense line of the myelin sheath (Readhead & Hood, 1994). The DM20 splice product of myelin proteolipid protein gene is the primary protein component of myelin (Regis *et al.*, 2002).

RNA was extracted using PureZOL RNA Isolation Reagent (Bio-Rad, Hercules CA, USA) and NucleoSpin RNAII (Machery-Nagel, Allectown PA, USA). cDNA templates were constructed through iScript Reverse Transcription kit (Bio-Rad, Hercules CA, USA). Target cDNA for caspase-3, MBP, or PLP/DM20 and reference target glyceraldehyde-3-phostphate dehydrogenase (GAPDH) were amplified using SsoAdvanced SYBR Green Supermix in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules CA, USA). Assays were run in sets of three for consistency. Amplification conditions were 95˚C for 30 seconds and 40 cycles of PCR, denaturation at 95˚C for 5 seconds, and annealing and/or extension for 30 seconds (60˚C for caspase-3, 57.5˚C for MBP and PLP/DM20). Comparative Ct method was used to analyze data.

Immunohistochemistry and Structure Quantification in the vmPFC:

Perfused brains were frozen on dry ice and sliced on a cryostat to a thickness of 50µm. Any sections containing the prefrontal cortex (PFC) were collected in intervals of three to allow three sets of PFC-containing sections to be used for later immunohistochemistry. Sections were

stored at -20˚C in cryoprotectant until staining on free-floating sections was performed. Brains perfused in adolescence 90 minutes following CMS procedure were used to stain for oligodendrocytes and neurons, while the myelination density analysis staining utilized mice perfused in adulthood following behavioral testing.

Three separate stainings were performed on gathered PFC-containing sections. An anti-NeuN antibody was used to identify fully differentiated, adult neurons as described in Wolf *et al.,* 1996. Olig2, an oligodendrocyte differentiation protein, was used to determine oligodendrocyte population (Zhou & Anderson 2002). Lastly MBP was utilized to show myelin density due to its structural use in production of the major dense line of the myelin sheath (Readhead & Hood, 1994).

The NeuN stain was an immunofluorescent stain using a primary antibody of mouse anti-NeuN (Chemicon #MAB377, Temecula, California, USA) at a 1:500 concentration and a secondary antibody of goat anti-mouse Alexa Fluor 555 (red) at a concentration of 1:500. Around six sections were used per animal; all steps proceeded on a shaker set to a low speed. Sections were washed in 1xPBS at room temperature, then were transferred to a blocking buffer. After being removed from the buffer, sections were incubated in the primary antibody overnight at 4°C. After this, the sections were again washed in 1xPBS, then incubated in the secondary antibody. From this point on all steps were set up with foil blocking the sections from excess exposure to light. Sections were washed again, after which they were mounted on a microscope slide and allowed to partially dry in a dark room. DAPI mounting media was used to preserve them and the slides were stored away from light exposure at 4°C until ready for counting. An example of this stain can be found in (Figure 2B).

Both the stain targeting oligodendrocyte somas, anti-olig2 (EMD Millipore ab9610, Darmstadt, Germany) and the stain targeting the myelinating projections, anti-MBP (ABCam 40390, Cambridge, MA, USA), utilized a diaminobenzidine (DAB) stain and vector lab biotinalated goat-anti-rabbit (Vector Laboratories B1000, Burlingame, CA, USA). The Olig2 stain utilized the primary antibody at 1:500 and secondary at 1:200 while the MBP stain had a primary antibody concentration of 1:200 and a secondary of 1:500. Around six sections of experimental prefrontal cortex were selected and washed in 1xPBS. Sections were then incubated in a hydrogen peroxide solution in PBS. A second set of washes were performed, then the sections were incubated in a blocking solution. Following this, the sections were transferred directly to a solution of the primary antibody in the blocker solution above at the aforementioned concentrations overnight at 4°C. The following day, the sections were washed again, then incubated in the secondary antibody in blocking buffer. After another wash, sections were transferred to an ABC solution (Vector Laboratories, Burlingame, CA, USA), washed again. A DAB solution was prepared including nickel sulfate for the Olig2 stain and omitting it for the MBP stain. Final staining proceeded until color matched a reference slide with all experimental sections incubating for the same length of time within the same stain. The DAB reaction was stopped in a sodium acetate bath. Lastly, sections were washed in TrisHCl (0.1 M). The experimental sections were then mounted, left to dry briefly, then preserved with Permamount solution. Staining example for each antibody in (Figure 1C and D) and (Figure 3A).

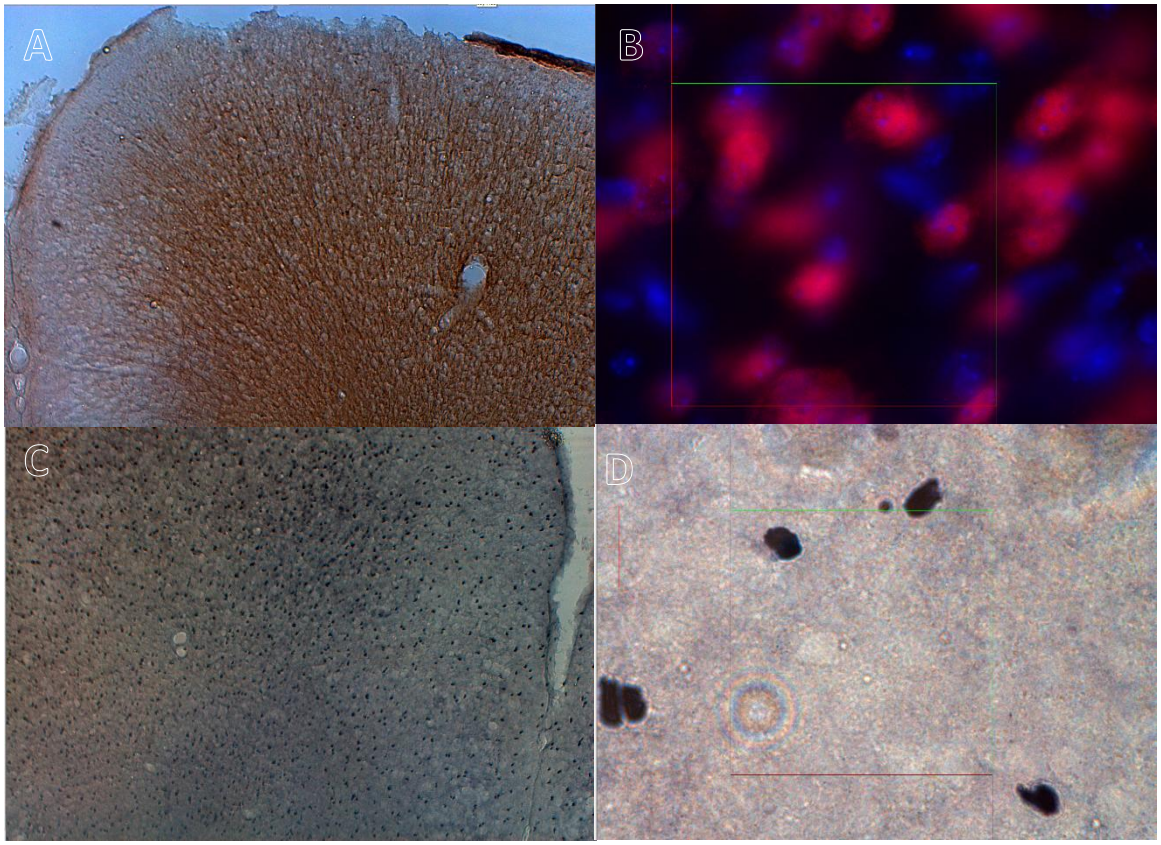

Figure 1: Microscopy photos. A: MBP-positive axons at 5x magnification labeled brown. B: NueN-positive cells at 63x magnification labeled red and blue for DAPI. C and D: Olig2-positive cells labeled black at 5x and 63x magnification respectively.

Using these slides, cell counting proceeded utilizing unbiased stereology methods by an experimenter blind to both the treatment and genotype of each subject. Neuronal and oligodendrocyte populations were quantified using the optical fractionator method and the Stereo Investigator cell-counting software from MBF Bioscience (Williston, VT, USA) (West *et al.*, 1991). Quantification was performed on every 3 sections with a total of three section per subject. The prelimbic and infralimbic cortexes of the ventromedial PFC were outlined at 5x magnification based on the Franklin and Paxinos mouse brain atlas, and positive cells from each stain counted at 63x magnification, oil immersion. The coefficient of error of Gunderson was kept below 0.10 for all subjects as defined by (Gunderson *et al.*, 1999).

ImageJ software was used to quantify myelination density. In this, grey scale level of 0 denotates black and 255 white. The mean greyscale level over the area of interest was analyzed and compared against the ambient light through the unimpeded slide and unstained tissue. The formula used to determine optical density is as follows:

$$\text{optical density} = \log_{10}\left(\frac{\text{incident light}}{\text{transmitted light}}\right)$$

This inverts the grey scale so that a dark signal results in a high value. The background OD was subtracted from the OD of each region of interest.

Attention Set Shift Task (ASST):

The Attention Set Shift Task was used to identify prefrontal-dependent cognitive impairments resultant from a genotype and stress interaction. Specifically, key parts of the test focused on identifying cognitive flexibility. Full detailed procedure can be found in (Heisler *et al.,* 2015). All behavioral procedure was conducted during the dark cycled of the reversed day and night cycle of the mice and any testing procedure occurred after a 1 hour acclimation period to the behavioral testing hall. The procedure began with a period of four days of handling (around 1 minute per day) and weight tracking. After this was a period of three days wherein mice were food restricted to help motivate food reward seeking within the test; body weight was kept above and around 85% of normal with a base amount of food being ~2g of standard food and two pieces of food reward, modulated based on weight percentage. This was maintained throughout the remainder of the procedure and any food reward acquired through the other phases of testing was deducted from their food restriction diet. At this point two ramekins were introduced to the mice's cages to allow acclimation to the testing material. This is also where

food was given. One gram of standard food and one piece of food reward was placed in each ramekin during feeding to avoid development of a side bias.

Following this was two days dedicated to acclimation to the testing arena. An example of the arena and the ramekins used can be found in (Fig. 2). The mice were given one hour in the arena with the ramekins from their cages as well as a ramekin for water. Some bedding from their home cages was added to give the arena a "home" smell. A food reward was placed in each ramekin with replacements added every 5 minutes while the mouse roamed. At all times the experimenter was visible. The arena was cleaned with 70% ethanol between subjects.

The next day was focused on training the mouse to dig in the filled ramekins. The initial setup was the same as the acclimation day, but every time that the mouse found the food rewards from each ramekin, it was reset in the holding area of the arena. This process was repeated at least twice more to ensure that the mouse was motivated to seek the reward, at which point the ramekins were filled to quarter volume with clean bedding and the process was repeated. After at

Media	**Scent**
Shredded paper	Vanilla
Sawdust	Orange
Gown	Rosemary
Felt	Lemon
Straws	Clove
Cotton balls	Fennel
Tile-x's	Ginger
Crinkle paper	Cinnamon
Raffia	Nutmeg
Critter confetti	Thyme
Paper streamers	
Ribbon	

Table A: List of possible media and scent available for ASST. Combinations were randomized.

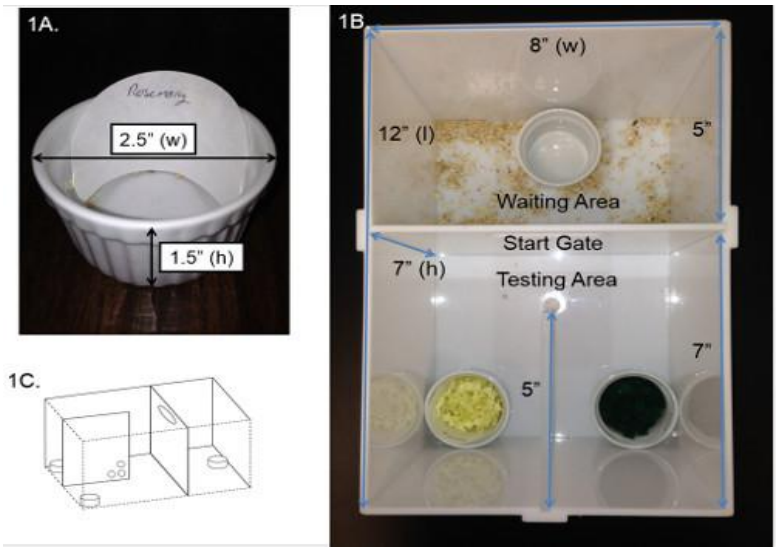

Figure 2: Example and dimensions of the ASST area and ramekins as utilized in the experimental procedure. From (Heisler et al., 2015)

least three successful and relatively quick trials, the volume of bedding was increased incrementally to half, three quarter volume, then full.

Assuming the subject passed the training day, testing proceeded over the course of the following two days. The subject was given a piece of food reward prior to testing to alleviate a hunger-based lack of focus during the test. The testing arena was set up as in (Fig. 2) with a clean ramekin for water. Each arm of the arena contained a ramekin with a material and/or scent depending on which test and group the mouse was in taken from (Table A). Each test consisted of a correct and incorrect ramekin based on burying material or scent that the mouse would have to determine and retrieve the food reward from for eight consecutive trials to pass –refusal to choose within three minutes resulted in a denotation of "no choice" and six consecutive refusals to participate or a failure to achieve the required eight consecutive correct trials in a row within fifty trials resulted in a failure and prohibited the mouse from moving on the next stage of testing. The layout of tests is presented in (Table B). The pairing of scent and media was randomized for each test and the arm position and confound pairing within the test was

Day	Test	Parameters
1	Simple Discrimination (SD)	Correct choice is either discrimination between two burying medias or two scents in clean bedding.
1	Compound Discrimination (CD)	Confounding dimension added; scent if in media group, media if scent. Original dimensions from SD kept.
1	CD Reversal (R1)	Correct choice is swapped with incorrect from SD.
1	Interdimensional Shift 1 (IDS1)	All dimensions changed out. Correct dimension still the same.
2	Interdimensional Shift 2 (IDS2)	As above.
2	Interdimensional Shift 3 (IDS3)	As above.
2	IDS3 Reversal (R2)	Reversal of IDS3 as before.
2	Extradimensional Shift (EDS)	Dimensions are changed out. Correct dimension is swapped; scent to media and vice versa.

Table B: List of tests given to ASST mice. The day of testing that the given test took place is given along with the name and parameters of the test.

randomized for each trial.

Each group is currently possessing an n=5-9. We are in the process of adding mice to get each group to an n=8-9.

Statistical Analyses:

All results are represented by mean values and standard error of the mean (SEM). Analyses were carried out using Prism (Graph-Pad Software Inc., CA, USA). RT-qPCR data was analyzed through the comparative ct method, with significance determined through a t-test. Immunohistochemistry and ASST data were analyzed through a 2 by 2 ANOVA test with genotype and exposure to CMS as independent variables. Structure analysis was done separately for the prelimbic and infralimbic vmPFC (PLC and ILC) as well as for the total for the whole vmPFC. Data was considered significant at $p \leq 0.05$.

Results

Quantification of Biomarkers and Markers of Cell Apoptosis in the PFC:

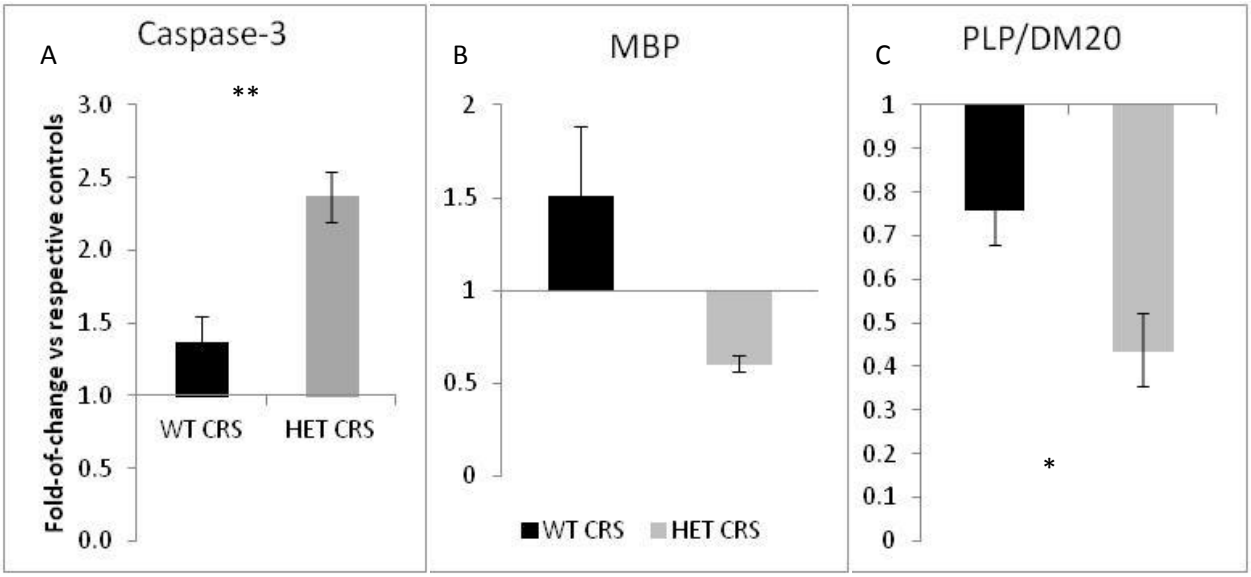

*Figure 3: mRNA levels of caspase-3 (A), MBP (B), and PLP/DM20 (C) in the PFC. In each graph, the treatment group is normalized to its respective control. Wildtype (WT) and heterozygous (HET) reared in control conditions (Control-WT n=4; HET n=4) or exposed to chronic mild stress (CMS) during juvenile development (CMS-WT n=4; HET n=5). CMS and genotype effect caspase- and PLP/DM20 levels *p<0.05; **p<0.01.*

mRNA expression of markers in the PFC were assessed in WT and HET control and CMS mice (Fig. 3). A significant stress effect was found in HET compared to WT mice for caspase-3; mRNA expression was greatly increased (p=0.0055) (Fig. 3A). For MBP (Fig. 3B), there was a trend nearing significance comparing the HET genotype to WT for stress exposure towards some reduction in mRNA (p=0.056). In PLP/DM20 (Fig. 3C), there was a significant reduction effect for HET mice compared to WT with stress exposure (p=0.031).

Immunohistochemistry and Structure Quantification in the vmPFC:

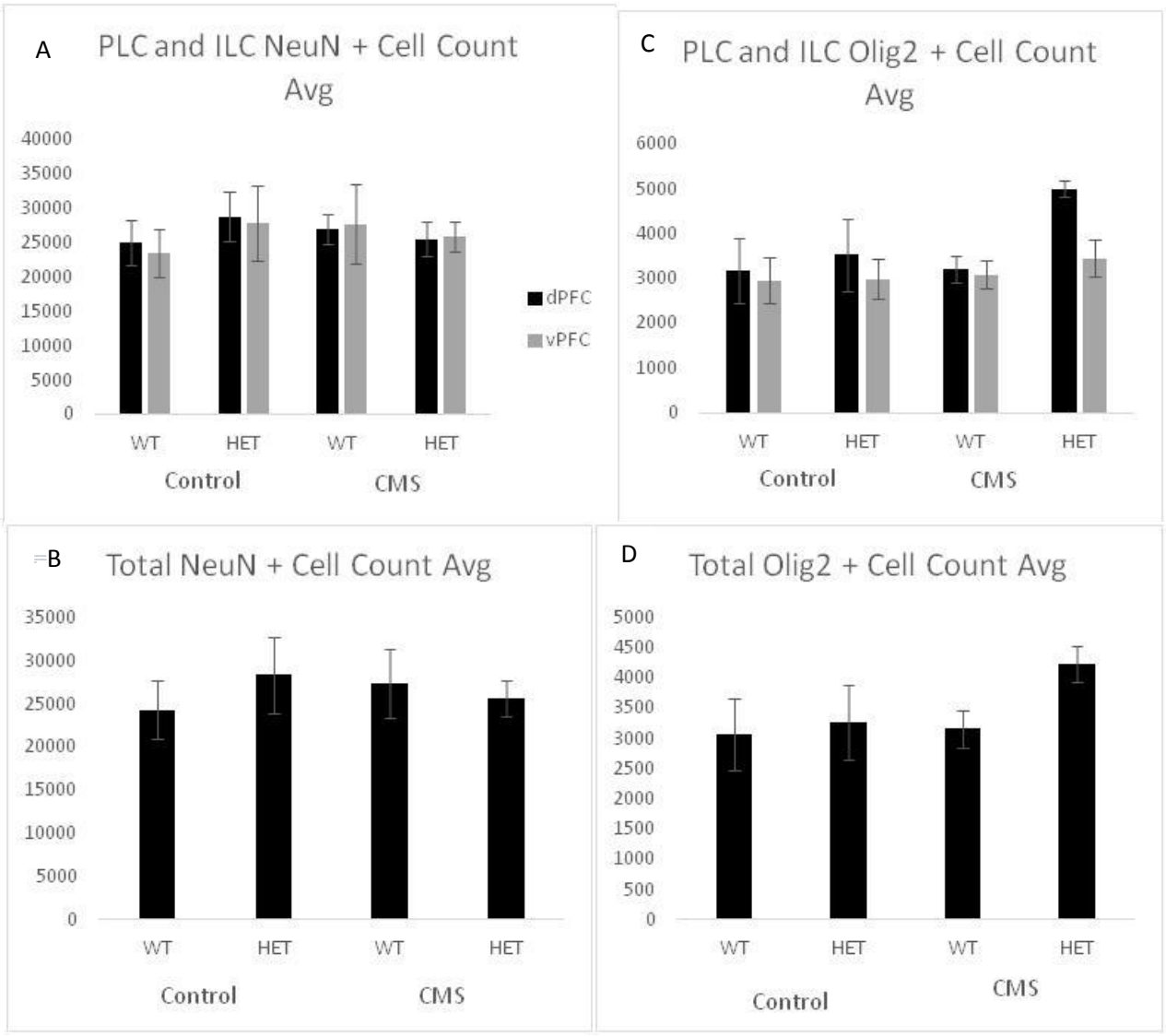

Figure 4: Average antibody-positive cells in the PLC, ILC, and overall for NueN (A and B) and Olig2 (C and D). Wildtype (WT) and heterozygous (HET) reared in control conditions (Control-WT n=4; HET n=3) or exposed to chronic mild stress (CMS) during juvenile development (CMS-WT n=3; HET n=3).

There was no main stress or genotype effect for the NeuN stain for the PLC, ILC, or overall, neither was there an interaction effect (Fig. 4A and B). For the Olig2 stain, no main stress or genotype effect was found, nor was an interaction detected (Fig. 4C and D).

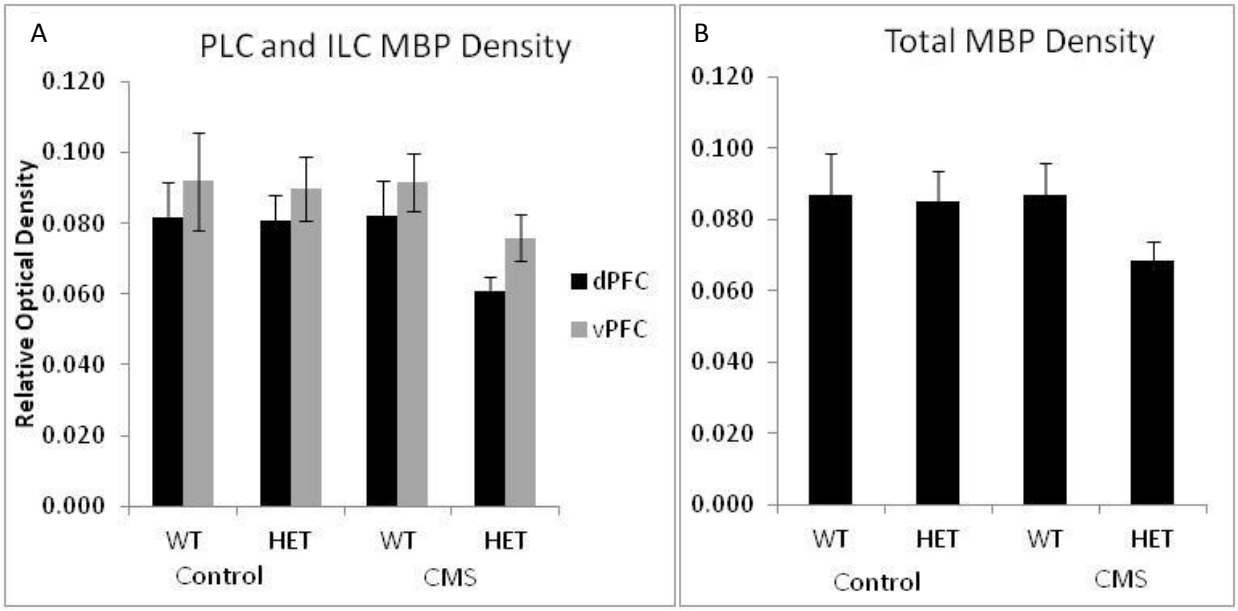

Figure 5: Average density of stained MBP in the PLC, ILC, and overall (A and B). Wildtype (WT) and heterozygous (HET) reared in control conditions (Control-WT n=4; HET n=4) or exposed to chronic mild stress (CMS) during juvenile development (CMS-WT n=3; HET n=5).

There was no main stress or genotype effect for MBP density in the PLC, ILC, or overall. We were unable to identify any interaction effect either; though the HET CMS group appeared to demonstrate some minor reduction, the p-value did not reflect any significant change (p=0.42).

Attention Set Shift Task (ASST):

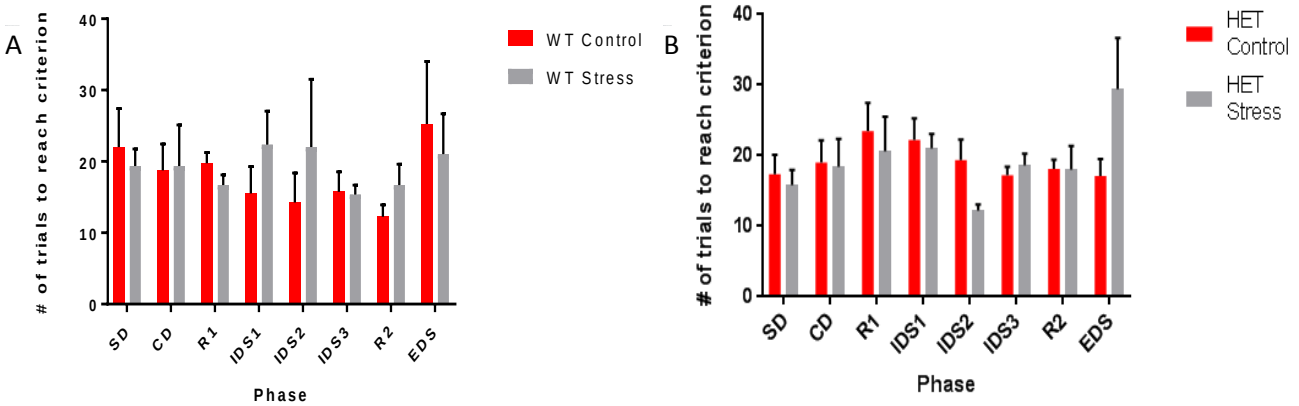

Figure 6: Average number of trials in each test to reach criterion separated by genotype (A and B). Wildtype (WT) and heterozygous (HET) reared in control conditions (Control-WT n=4; HET n=9) or exposed to chronic mild stress (CMS) during juvenile development (CMS-WT n=3; HET n=5).

There was no stress effect detected for WT mice. In the HET group, a stress exposure trend was identified in the extradimensional shift (EDS) trial ($p=0.078$) (Fig. 6B). As of right now, additional animals still need to be tested to improve the power of the analysis and an outlier exists that skews the data out of significant range –if removed, the data drops below a p of 0.05, so expectations are still that the data will display significance once the last of the mice are tested.

Discussion

In this study, we demonstrated that adolescent chronic mild stress produces effects in specific myelin-related biomarkers MBP and PLP/DM20 and the apoptosis-involved marker caspase-3 (Fig. 3) in HET Npas4 expressing mice. We also demonstrated a trend towards impairment in the attention set shift task for HET mice, specifically in the extradimensional shift test that is described by (Heisler *et al.,* 2015) as one of the stages requiring cognitive flexibility that is restricted in instances of PFC-related impairment. Additional mice have yet to be tested and were there not an outlier, the data would be significant. Therefore, it is reasonable to conclude that this form of adolescent mild stress in Npas4 deficient mice has the potential to induce cognitive impairment that is similar to symptoms found in various psychological disorders that produce similar results in the original human analog of this test. Cognitive flexibility is described as the ability to switch attention between task rules or sets as in the Wisconsin card sorting task. Interestingly, several distinct frontal cortex regions are involved in variations of switching in related tasks including the dorsolateral PFC, medial PFC. There is also a trend towards involvement with continuously more complex functions as regions lie further posterior in the PFC (Kim *et al.,* 2011).

Adolescence is a key point in development for the brain and especially for the PFC. Exposure to stress during this timeframe has been shown to have profound effects on behavior and on development of psychological disorders in adulthood (Buwalda *et al.,* 2011). Recent work in our lab has demonstrated that stress exposure and Npas4 expression interact to produce significant behavioral changes, and additionally that adult stress has a much less severe effect on behavior compared to stressors in adolescence (Coutellier *et al.,* 2015). It is possible that chronic

stressors could affect Npas4 expression, dropping it below functional level and leading to the impaired phenotype.

Although the trend in behavior represented potential stress-related abnormalities in the makeup of the PFC –despite the biomarkers of myelin structure being significantly downregulated and caspase-3 demonstrating more than a twofold increase in stress-exposed Npas4 deficient animals –no significant change in the number of neurons (NeuN-positive) the number of oligodendrocytes (olig2-positive) nor in overall myelin density (MBP-positive axons) was found in the ILC or PLC (Fig. 4 and 5). This could indicate that the vmPFC is an unaffected region of the PFC in instances of Npas4-mediated stress changes, that structural changes that were not considered are present (such as myelin thickness or dendritic spine density), or that the power of the analysis was too low due to low ns throughout the study.

As mentioned before, a large portion of the PFC can be linked to these impairments. (Kim *et al.,* 2011) specifically identifies the lateral and medial frontopolar (LFPC and MFPC) and the inferior frontal gyrus (IFG) in being directly involved with the type of cognitive set switching that we were interested in here. It is possible that the incongruence between the RT-qPCR data and the vmPFC structure quantification data is due to the change in structure being in a separate, distinct section such as the FPC or IFG. These prefrontal cortical areas are compelling targets in which to look for instances for reduced neurons and myelin.

Alternatively, these results could indicate that changes in these markers relate not to a reduction in number of myelinated processes, but to a reduction in the quality of the structures instead. Lui *et al.* (2012) demonstrated, through electron micrographs, reduced myelin thickness in the PFC following social isolation of mice in adulthood, despite no changes in oligodendrocyte populations. This is supported by the results produced through our analysis of

oligodendrocytes following CMS. It is possible that a change in myelin thickness is enough to elicit the mRNA expression and behavioral results collected, though this change has only been associated with social withdrawal thus far and is also reversible with the model they used. Potentially, this change in the ratio of myelin thickness to axon diameter could slow proper signal transduction speed; this effect becomes more significant as the axon length increases (Rushton, 1951).

Despite there being a several-fold increase in the PFC of caspase-3, a protein commonly considered an integral part of the apoptosis process, no quantifiable cell death was detected in our data (Porter & Jänicke, 1999). While caspase-3 is necessary to carry out specific aspects of cell apoptosis such as DNA fragmentation, it also serves non-apoptotic functions in differentiation during neurogenesis –including astrocyte and oligodendrocyte differentiation – and plays a role in synaptic plasticity and pruning (D'amelio *et al.,* 2009). This suggests that increased caspase-3 levels without obvious cell death may be associated with overabundant dendritic spine or axonal pruning. Since Npas4 modulates inhibitory synapse formation, this may provide a mechanism by which Npas4 contributes to caspase-3 activity and changes the extent that it prunes dendritic spines during adolescence. This could explain how such high levels of what is generally considered a protein component of the cell death mechanism could occur without obvious changes in cellular populations.

The original research question produced answers that we did not anticipate. No truly significant data regarding cognitive flexibility in this Npas4-deficient CMS model was determined –though previous work has demonstrated a more certain correlation –nor were any changes identified in the structures we analyzed in the regions we focused on. Specific markers in the overall PFC provide compelling directions in which to move forward in future projects,

though the implications of the differences in concentrations of myelin-related markers and caspase-3 in terms of how they relate to changes in this area remain largely unknown at this moment. Our results imply that any interaction between Npas4 and adolescent stress does not produce loss of neurons or changing in myelination density, at least in the ventromedial portion of the PFC. Understanding how Npas4 modulates the effects of adolescent stress exposure on the PFC related to the occurrence of cognitive impairments remains important. Breakthroughs here may help in uncovering the mechanisms and causes behind human neurological disorders forming in adolescence and moving to prevent or treat them more efficiently.

Conclusion

Adolescent stress has the potential to interact with specific genetic factors, including expression of Npas4, to produce significant cognitive and behavioral impairments important in their relation to various psychological disorders. While we cannot conclude any significant reduction in cognitive flexibility in this study, data is trending towards PFC-mediated function impairment through ASST. Additionally, markers classically associated with myelin structure were found to be reduced in the overall PFC, along with greatly increased expression of caspase-3 –an important protein involved with cell death. Although this trend seems to indicate some change in structures present in the PFC, analysis of neurons, oligodendrocytes, and myelination in the PLC and ILC of the vmPFC have not demonstrated any noticeable change. Still, a reduction in Npas4 expression has been demonstrated to alter responses to stress, especially with exposure to stress in adolescence, and produce changes in PFC-reliant behavior that cooccurs with alterations in the hippocampus (Coutellier *et al.*, 2015). This suggests that the PFC is also undergoing changes that have yet to be identified, if not in parameters scrutinized here, then in other specific areas of the PFC or in other aspects that we did not consider.

Recommendations

Further inquiry is required to understand how the significant changes in protein concentrations in the total PFC relate to alterations in specific regions therein. Considering neuron and oligodendrocyte populations or myelination in other areas of the PFC besides those in this study could identify changes that are not present in the vmPFC where we looked. One such potential target for this is the FPC due to its involvement with cognitive flexibility functions and its presence in mouse models. Since this model of CMS and Npas4 deficiency has also demonstrated anxious and depressive behaviors, other areas that are associated with these types of behavioral changes are also viable. Additionally, quantifying myelin thickness in these areas of the PFC could reveal novel changes that were not detectable through our analysis of overall density. Beyond this, analysis of dendritic spines, already demonstrated to be altered in some stress models, could reveal a potential interaction of Npas4 and caspase-3 that causes excessive synaptic pruning unrelated to full-fledged cell death. It is also possible that more severe, unpredictable forms of chronic stress could be necessary to reveal subtler structural changes. Modifying the CMS procedure and reproducing these tests could illuminate differences that were hidden by small relative differences or acclimation to the stressor.

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